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Carole B Christian

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13. ABSTRACT ***(Maximum 200 Words)***
The overall goal of this project was to develop a clinical-grade serum-free medium that specifically supported dendritic cell (DC) formation and antigen presentation. In two separate series of experiments we have characterized a serum-free medium that supports the ex vivo proliferation of DCs as well as their antigen presenting ability. In the first series of experiments we used $CD34^+$ cells derived from mobilized peripheral blood stem cells and differentiated them to DCs in the presence of the cytokines GM-CSF, IL-4 and TNFα, although the serum-free medium supported this process it yielded only a small percentage of the DCs (3-5%)that are too few in number for clinical protocols. In a second series of experiments we used this same QBI serum-free formulation and differentiated DCs from normal monocytes that yielded over 80% of the population positive for the DC immunophenotype. This compared nearly equivalent in both number and antigen presenting ability to a proprietary serum-free medium developed specifically for lymphocyte proliferation and used in DC clinical trials. Future experiments are designed at optimizing this medium and evaluating other nutritional factors, cytokines and culture conditions to yield optimal DCs both in terms of number and function.

14. SUBJECT TERMS
Ex Vivo, serum-free medium, cell culture, dendritic cells, breast cancer

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INTRODUCTION:

The goal of this study was to develop clinically relevant culture conditions that support ex vivo expansion of dendritic cells (DCs) as well as their function in antigen presentation. Serum contains numerous unknown and ill-defined components as well as the potential to contain adventitious agents. The use of serum-free components can circumvent many of these difficulties. Presently the only serum-free media used to culture DCs was developed for other cell types and not specifically for DC proliferation/differentiation. The development of a clinical-grade serum-free medium specifically for DCs is necessitated by the technological difficulties in generating adequate numbers of highly purified DCs.

BODY:

Our initial experiments involved culturing $CD34^+$ cells from mobilized peripheral blood. Peripheral blood progenitor cells (PBPCs) from mobilized breast cancer patients were purchased from Bio-Whittaker (Walkersville, MD). The $CD34^+$ cells were isolated using Isolex-300i columns (Baxter, Inc., Deerfield, Illinois). The cells were assayed for $CD34^+$ purity and are typically in the range of 90-95% pure. The $CD34^+$ (2×10^5 ml) were cultured for 2-3 weeks at 37°C, in a fully humidified incubator containing 5% CO_2 and air, in either alpha-modified Eagles medium (αMEM) or Iscove's modified Eagel's medium (IMEM) plus 10-20% fetal bovine serum (FBS) or QBI's initial serum-free medium comprised of Iscove's modified Dulbecco's medium (IMDM) plus 4 mg/ml HSA, 300 µg/ml human transferrin, and 0.48 U/ml human recombinant insulin. Each media was supplemented with 50 ng/ml GM-CSF, 25 ng/ml IL-4 and 10 ng/ml tumor necrosis factor-α (TNF-α) (R&D Systems, Minneapolis, Minnesota). Cells were harvested as noted and analyzed for dendritic cell surface markers. Tables I and II represent data from flow cytometric analysis of three separate experiments illustrating that the serum-free culture system was able to support the differentiation of $CD34^+$ cells to cells expressing the dendritic markers, especially $Lin^-/DR^+/CD11c^+$ and $Lin^-/DR^+/CD80^+$.

Table I. Two separate experiments comparing dendritic cell differentiation in alpha-MEM with 20% FBS compared to QBI's initial serum-free formulation. Each media contained the cytokines: GM-CSF, IL-4 and TNF-α.

		Alpha-MEM + 20% FBS (1×10^6 cells)					QBI Initial Formulation (1 x 106 cells)			
Markers	Expt	Day 7	Day 10	Day 14	Day 17	Expt	Day 7	Day 10	Day 14	Day 1
Lin-/DR+/1a+	1	11.1	18.7	4.7	3.5	1	1.5	0.1	1.3	0.4
	2	8.6	17.6	7.7		2	0.2	0.4	0.9	0.8
Lin-/DR+/11c+	1	10.1	19.5	10.2	12.9	1	0.3	1.5	7.2	3.6
	2	7.8	23.2	16.6		2	0.2	2.0	7.2	4.5
Lin-/DR+/40+	1	10.2	27.8	10.6	12.9	1	0.9	1.2	5.9	2.6
	2	7.1	32.0	16.7		2	0.5	2.3	6.5	3.4
Lin-/DR+/80+	1	1.3	10.7	3	2.8	1	0.02	0.1	0.5	0.4
	2	0.5	9.5	4.5		2	0.01	0.3	0.4	0.5
Lin-/DR+/83+	1	0.5	5.8	0.6	0.8	1	0.001	0.01	0.1	0.03
	2	0.2	5.2	1.1		2	0.004	0.08	0.2	0.06
Lin-/DR+/86+	1	2.6	15.8	3.3	2.2	1	0.1	0.3	1.2	0.04
	2	1.7	14.1	4.4		2	0.02	0.5	1.2	0.07

Table II. A single experiment comparing dendritic cell differentiation in IMDM with 10% FBS compared to QBI's initial serum-free formulation. Each media contained the cytokines: GM-CSF, IL-4 and TNF-α.

IMDM+10% FBS (Total Cell Number x 10^5)				QBI Initial Formulation (Total Cell Number x 10^5)		
Markers	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
Lin-DR+1a+	2.1	5.0	1.9	0.6	1.4	0.0
Lin-DR+11c+	15.5	16.8	3.8	4.3	4.5	3.6
Lin-DR+40+	0.2	0.0	0.8	0.3	0.2	0.0
Lin-DR+80+	3.1	6.3	4.4	1.1	2.0	0.54
Lin-DR+83+	0.1	0.1	0.2	0.1	0.2	0.0
Lin-DR+86+	9.1	10.2	5.9	4.3	5.4	2.1
Lin-DR+123+	42.7	8.6	7.6	17.0	5.6	1.1

In a third experiment we evaluated the expansion and differentiation of mobilized peripheral blood $CD34^+$ cells to functional dendritic cells. Again, peripheral blood progenitor cells (PBPCs) from mobilized breast cancer patients were purchased from Bio-Whittaker (Walkersville, MD). The $CD34^+$ cells were isolated using Isolex-300i columns (Baxter, Inc., Deerfield, Illinois). The cells were assayed for $CD34^+$ purity and are typically in the range of 90-95% pure. The $CD34^+$ (2×10^5 ml) were cultured for 2-3 weeks at 37°C, in a fully humidified incubator containing 5% CO_2 and air, Iscove's modified Eagel's medium (IMEM) plus 10% fetal bovine serum (FBS) or QBI's initial serum-free formulation. Each media was supplemented with 50 ng/ml GM-CSF, 25 ng/ml IL-4 and 10 ng/ml tumor necrosis factor-α (TNF-α) (R&D Systems, Minneapolis, Minnesota). In both experiments cells were harvested after 3 weeks and analyzed for dendritic cell surface markers, Table III and IV. In both experiments the dendritic markers, $Lin^-DR^+CD11c^+$, had increased in serum containing media to 31.2×10^5 and 4.4×10^5 cells, respectively and in the QBI's initial serum-free formulation to 10.2×10^5 and 14.0×10^5 cells, respectively. Such data again indicates that both culture systems supported DC maturation, although, it takes 7 to 10 days longer in the serum-free medium as previously reported.

Table III

IMDM+ 10% FBS (Total Cell Number x 10^5)		Initial Formulation (Total Cell Number x 10^5)
Markers	Day 22	Day 22
Lin-DR+1a+	1	0.8
Lin-DR+11c+	31.2	10.2
Lin-DR+40+	0.2	2.4
Lin-DR+80+	1.9	8.9
Lin-DR+83+	0	1.5
Lin-DR+86+	3.5	11.6
Lin-DR+123+	10.7	1.2

Table IV

IMDM+ 10% FBS (Total Cell Number x 10^5)		Initial Formulation (Total Cell Number x 10^5)
Markers	Day 25	Day 25
Lin-DR+1a+	0.2	1.4
Lin-DR+11c+	4.4	14.0
Lin-DR+40+	0	0.5
Lin-DR+80+	0	9.9
Lin-DR+83+	0	6.9
Lin-DR+86+	.01	11.6
Lin-DR+123+	0.5	2.4

Tables III and IV: Two separate experiments comparing dendritic cell differentiation at the noted culture times in IMDM with 10% FBS versus QBI initial serum-free formulation each media contained the cytokines: GM-CSF, IL-4 and TNF-α.

The dendritic cells from this last experiment (Table IV) were evaluated in a mixed allo-lymphocyte assay. Briefly, lymphocytes (1 x 10^5/0.1 ml) were cultured with different dilutions of irradiated (1500 RAD) dendritic cells (beginning at 1 x 10^5/0.1 ml) in IMDM + 10% AB serum for six days. At the end of this time frame we added 0.7 μC; H^3-thymidine to each well and incubated an additional 12 hours, then counted. Figure 1 represents the fold stimulation using the DCs cultured in serum versus the initial serum-free formulation. The DCs cultured in serum-free medium yielded a typical stimulation index illustrating their ability to function in an MLR assay. The DCs cultured in serum containing medium did not have a significant stimulation index, most likely because DCs cultured in serum mature earlier, by day 14 of culture. In conclusion we have developed culture systems that yield DCs and determined that those cultured in serum-free medium for 3 weeks can support a mixed lymphocyte reaction.

Because relatively large yields of DCs can be derived from monocytes and can be easily obtained from patients/donors, we began the evaluation of our culture conditions for expansion of DCs using $CD14^+$ selected or adherent cells from normal, healthy consented peripheral blood mononuclear cell (PBMC) volunteers. Adherent cells or $CD14^+$ immunoselected (Miltenyi, CA) monocytes were collected from apheresis PBMC units (COBE Spectra, 9.3±2.2x10^9 WBC, n=4) or whole blood buffy coats and cultured at 37°C for 7-8 days in XVIVO 15 (Biowhittaker, MD) or QBI's initial serum-free formulation containing cytokines. There are few serum-free media available for DC culture. XVIVO 15 media specifically formulated for macrophages and lymphocytes is currently being used in several protocols so was selected as a standard for DC production. Purity of $CD14^+$ monocytes was >96% judged by flow cytometry ($CD14^+$, $CD3^-$, $CD19^-$, $CD56^-$, BD Biosciences Pharmingen, CA) (FACscan, Becton Dickinson, CA) and differential analysis (CellDyn, Abbott, IL). Adherent fractions were comprised mostly of $CD14^+$ monocytes (71±10.6%). The cytokines GM-CSF (25 ng/ml) and IL-4 (25 ng/ml) (Pharmingen, CA) alone were added on day 0. To induce maturation TNF-α (30 ng/ml) was added to matching cultures on day 4. Both opened (6-well polystyrene culture plates) and clinical, closed (i.e., (Nexell Therapeutics, CA) culture bags using sterile-docking) systems were tested. Following the culture period, cells were harvested and assessed for viability using trypan blue exclusion and annexin-propidium iodide by flow cytometry (Coulter-Immunotech, FL). DC phenotype (i.e.,

flow analyses of surface markers CD1a, CD11c, CD14, CD80, CD86, CD83, MHC class I, II, mannose receptor, ILT3 and function (i.e., mixed lymphocyte response (MLR), antigen presentation) were also assessed. Table V shows high viabilities of cells after 7 days in culture without any added serum demonstrating that cells can be maintained under these conditions. QBI's initial serum-free culture formulation is able to support the differentiation of $CD14^+$ monocytes to DCs. The cells expressed an "immature" phenotype, ($CD14^-CD80^+CD86^+DR^+$), following culture in GM-CSF+IL-4 and a "mature" phenotype by the appearance of $CD83^+$ cells in cultures with GM-CSF+IL-4+ TNF-α added on day 4. The $CD83^+$ expression levels differ between the media. It is not yet understood how these markers correlate with function; however, serum-free-cultured cells are able to stimulate and present antigen to T-cells despite different levels of expression as shown in Table VI.

Table V. Viability and Phenotype

Viability	Cytokine Treatment		
(n=4 per condition)	GM-CSF+IL4	GM-CSF+IL4+TNFα	No growth factors
XVIVO15	92.4±7	82±11.8	90±20
Initial Formulation	90±9	93±10	99±3
Total mean based on media type (n=12 per media type): XVIVO15=88±13; Initial Formulation= 93±8 (ANOVA; p=0.208)			
Phenotype: Cells cultured in GM-CSF+IL4 expressed high levels (>80%) CD1a, CD11c, CD40, CD80, CD86, HLADR, HLAABC in both media. GM-CSF+IL4+TNFα cultures also expressed the maturation marker CD83 (XVIVO15 85±8% and QBSF 40±22%)			

Table VI. Function

	XVIVO 15		***Initial Formulation***	
Cytokine Treatment	*MLR **	*Stimulation Index***	*MLR*	*Stimulation Index*
No growth factors	6,833 ± 2,498	3±3	7,766 ± 1,934	2 ± 2
GM-CSF+IL4	28,635 ±14,489	9.2±2	33,727 ± 9,999	5.3 ± 2
GM-CSF+IL4+TNF□	43,085 ± 13,510	6.5±2	34,320 ± 15,327	4.5 ± 2
Total values based on media type (n=12 per media type)	25,271 (SE=5865)	6.2 ± 3	26,184	4 ± 1.7
(n=4 per condition) *MLR=allogenic lymphocyte response-values are given as counts per minute (CPM) measured on gamma counter (Packard Instruments, CT) via uptake of 3H-thymidine after 5 day coculture of graded DC amounts from10^5 (shown) against 10^5 T cells **Stimulation Index= Antigen presentation of tetanus toxoid (tet 2ug/ml) shown as CPM of DCs+ tet/DCs-tet				
STATISTICS- MLR: ANOVA, p=0.461 Tukey; Antigen presentation: ANOVA, p=0.474				

ALLO-MLA USING HUMAN DENDRITIC CELLS DIFFERENTIATED IN QBI's INITIAL SERUM-FREE FORMULATION OR IMDM WITH 10% FBS MEDIUM

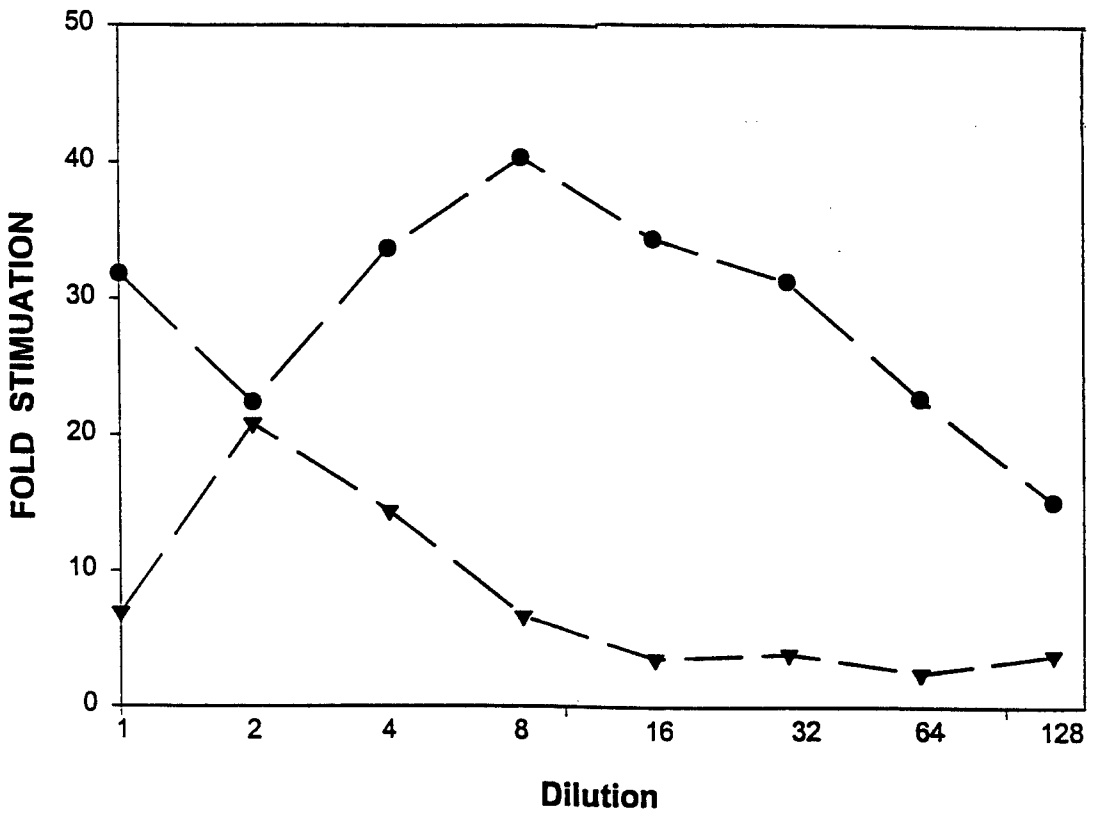

Figure 1. DCs cultured in either serum (triangles) or serum-free medium (circles) then cultured with fresh lymphocytes (1 x 10^5 per 0.1 ml) and varying dilutions of DCs (beginning at 1 x 10^5 per 0.1 ml) in IMDM + 1-% AB serum. After six days of co-culture they were pulsed with H^3-thymidine for 12 hours and counted

KEY RESEARCH ACCOMPLISHMENTS:
A clinical-grade serum free formulation that supports DC formation remains feasible.

REPORTABLE OUTCOMES:
The work presented herein has been used to apply for funding – Idea Proposal, Log # BC021256, entitled "Delineation of Ex Vivo Culture Condition for Breast Cancer Dendritic Cell Immunotherapy". This proposal will build on the above findings to develop an optimal clinical-grade serum-free medium for DC formation.

CONCLUSIONS:
The data reflects the ability of the QBI initial medium to specifically support ex vivo DC formation as well as function. The ex vivo culture of DCs derived from $CD34^+$ cells is tenuous because of the low number of cells that have the DC immunophenotype. This is the case in both the serum-free media, as well as the serum containing medium suggesting that DCs formed from $CD34^+$ cells may be to few in number for clinical use. This situation appears not to be the case using monocyte derived cells in which 80% of the ex vivo cultured population has the DC immunophenotype. It is now feasible to use the later system evaluate other components as: lipids, cholesterol, fatty acids, and different fractions of HSA for their affect on DC formation and antigen presentation. Once such an optimal clinical-grade formulation is developed further evaluation of various cytokine mixtures and concentrations, as well as the length of culture times to obtain optimum DC formation and function can be pursued.

REFERENCES:
None

APPENDICES:
None

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